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# Different ecophysiological responses of freshwater fish to warming and acidification

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ABSTRACT

Future climate change scenarios predict threatening outcomes to biodiversity. Available empirical data concerning biological response of freshwater fish to climate change remains scarce. In this study, we investigated the physiological and biochemical responses of two Iberian freshwater fish species (*Squalius carolitertii* and the endangered *S. torgalensis*), inhabiting different climatic conditions, to projected future scenarios of warming (+3 °C) and acidification (ΔpH = −0.4). Herein, metabolic enzyme activities of glycolytic (citrate synthase - CS, lactate dehydrogenase - LDH) and antioxidant (glutathione *S*-transferase, catalase and superoxide dismutase) pathways, as well as the heat shock response (HSR) and lipid peroxidation were determined. Our results show that, under current water pH, warming causes differential interspecific changes on LDH activity, increasing and decreasing its activity in *S. carolitertii* and in *S. torgalensis*, respectively. Furthermore, the synergistic effect of warming and acidification caused an increase in LDH activity of *S. torgalensis*, comparing with the warming condition. As for CS activity, acidification significantly decreased its activity in *S. carolitertii* whereas in *S. torgalensis* no significant effect was observed. These results suggest that *S. carolitertii* is more vulnerable to climate change, possibly as the result of its evolutionary acclimatization to milder climatic condition, while *S. torgalensis* evolved in the warmer Mediterranean climate. However, significant changes in HSR were observed under the combined warming and acidification (*S. carolitertii*) or under acidification (*S. torgalensis*). Our results underlie the importance of conducting experimental studies and address species endpoint responses under projected climate change scenarios to improve conservation strategies, and to safeguard endangered freshwater fish.

## 1. Introduction

Earth's climate is changing at an unparalleled pace, threatening biodiversity worldwide (Field et al., 2014; Hartmann et al., 2013; Pörtner et al., 2014). In fact, air temperature is projected to increase between 2.6 and 4.8 °C (Collins et al., 2013) and atmospheric $CO_2$ concentration can reach values between 420 and 940 ppm by 2100 (Collins et al., 2013; Pörtner et al., 2014). Freshwater ecosystems are particularly at risk due to alterations in thermal and precipitation regimes which, in turn, will drastically change the dynamics between floods and droughts, decrease of river flow and increase of the risk of extreme events (e.g. heat waves) (Field et al., 2014; Füssel et al., 2012). Also, the increase in acid rainfall, resulting from emissions of sulfur dioxides and nitrogen oxides to the atmosphere, will contribute to the acidification of lakes and rivers (Leduc et al., 2013; Van De Waal et al., 2010). All of this will unquestionably pose further challenges for fauna living in these habitats (Leduc et al., 2013).

Freshwater fish, as ectotherms, strongly rely on environmental temperature in order to regulate their metabolism and may have a reduced migration ability, making them prone to warming conditions (Angilletta, 2002; Berg et al., 2010). Increasing temperature is even more alarming for those species living closer to their thermal tolerance limits (Reusch and Wood, 2007; Somero, 2010; Tomanek, 2010; Hoffmann and Sgrò, 2011). Even though many studies have approached the subject of thermal stress in freshwater fish (e.g. Podrabsky and Somero, 2004; Yamashita et al., 2004; Fangue et al., 2006; Jesus et al., 2013, 2016; Campos et al., 2017), only a few have attempted to study the effects under the context of climate change (e.g. de Oliveira and Val,

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2016; Mccairns et al., 2016; Prado-Lima and Val, 2016; Jesus et al., 2017, under review). Furthermore, the acidification of freshwater ecosystems have been poorly studied, despite the predictable effects that freshwater biota will suffer as a result of it (Leduc et al., 2013; Ou et al., 2015a). In fact, major focus has been given to ocean acidification and this process is widely known to affect many marine species physiology and behaviour (e.g. Munday et al., 2009a; Aurélio et al., 2013; Vinagre et al., 2013; Pimentel et al., 2016, 2015; Rosa et al., 2014, 2016).

The Iberian chubs, *Squalius carolitertii* (Doadrio, 1988) and *Squalius torgalensis* (Coelho et al., 1998), are two closely related endemic freshwater fish species, which inhabit two distinct regions with different climatic conditions (Carvalho et al., 2010): *S. carolitertii* inhabits the northern region of Iberian Peninsula, whereas *S. torgalensis* has a restricted distribution to the Mira river basin, in the southwestern region of Portugal (Coelho et al., 1998). While *S. caroltertii* lives under milder conditions, *S. torgalensis* is exposed to higher temperatures and greater temperature fluctuations, particularly during the dry season where individuals of this species are often confined to small ponds (Coelho et al., 1998; Magalhães et al., 2003). These two distinct climates expose these species to different seasonal and even daily water temperature fluctuations, which in turn result in different life history traits such as different life span, spawning age and body size (Magalhães et al., 2003). Additionally, previous works on gene regulation of both species suggest that *S. torgalensis* seems to be better adapted to higher temperatures, presenting higher survival rates and stronger responses in gene expression under high temperatures when compared to *S. carolitertii* (Jesus et al., 2016, 2013).

When facing stressful conditions, organisms may display several physiological responses to survive under the adversities. Adjustments in metabolic pathways are amongst the most common responses and may lead to shifts in energy production (Campos et al., 2017; Mwangangi and Mutungi, 1994). The activities of citrate synthase (CS) and lactate dehydrogenase (LDH) can reflect these modifications in aerobic and anaerobic potential, respectively, and thus represent good biomarkers for these metabolic pathways (McClelland et al., 2006). Another highly common response to stressful conditions is the heat shock response (HSR) (Morris et al., 2013; Wegele et al., 2004), which consists in the synthesis of a specific group of proteins (heat shock proteins (HSP)) that are responsible for the stabilization and refold of denatured proteins as a response to increasing temperatures (Dong et al., 2008; Fangue et al., 2006; Tomanek, 2010; Yamashita et al., 2004). In addition, the production of molecules that derive from oxygen, i.e. reactive oxygen species (ROS), (e.g. superoxide anion and hydrogen peroxide) (Sevcikova et al., 2011; Sun et al., 2007) is also a good indicator of stress (Sevcikova et al., 2011; Storey and Storey, 2005; Sun et al., 2007). ROS trigger the individual's antioxidant defense system by producing antioxidant enzymes, trying to reestablish the oxidant balance. However, in excess ROS situations, several biological features of the organisms may be damaged, including cellular health and integrity due to lipid peroxidation (Sevcikova et al., 2011).

The present study aims to understand the effects of warming plus acidification on the physiology of the Iberian chubs, *S. carolitertii* and *S. torgalensis*, inhabiting different climatic regions, by using conventional stress-related biomarkers (metabolic and antioxidant responses). Particularly, we investigated the combined effects of warming (+ 3 °C) and acidification (ΔpH = − 0.4), in relation to summer average parameters, on the metabolic potential (CS and LDH activities), heat shock response, antioxidant enzymatic machinery [glutathione *S*-transferase (GST), superoxide dismutase activity (SOD) and catalase (CAT)] and peroxidative damage [malondialdehyde (MDA)] of these two species.

This study provides important insights on the threats of climate change, a scenario presently considered irreversible to freshwater species (Collins et al., 2013). Moreover, since *S. torgalensis* is a critically endangered species (Coelho et al., 1998), this work is of utmost importance for surveying the threats that this species may face in future, in order to adopt proper conservation measures.

## 2. Material and methods

### 2.1. Sampling

*S. carolitertii* and *S. torgalensis* specimens were field collected in two river basins (Mondego: 40° 8′5.22″N–8° 8′35.06″W; Mira: 37°38′1.31″N–8°37′22.37″W), located in the west coast of Portugal. An electro-fishing device (300 V, 4A; Hans Grassl, Model EL 62) was used to perform fish collection, and the avoidance of juvenile mortality was accomplished by applying short duration pulses (3–6 ms). Organism sampling was performed during spring (May to June 2014), where water temperature and pH varied between 17.80 ± 0.67 °C and 8.08 ± 0.01 for Mondego river, and 19.50 ± 0.21 °C and 8.23 ± 0.02 for Mira river (measured with a YSI-85 handheld system). Capture procedures were performed under ICNF license (n° 263/2014/CAPT, Instituto da Conservação da Natureza e Florestas).

### 2.2. Experimental design

After collection, fish were transported in isothermal cases, under constant aeration conditions, to the Laboratório Marítimo da Guia (Cascais, Portugal). Subsequently, fish were progressively acclimated (2 weeks) to laboratory conditions, mimicking summer average values at the collection sites [data obtained from the national information system of water resources (http://snirh.pt) from 2002 to 2006] for temperature and pH under normoxic (8 $mg{\cdot}L^{-1}$). After this acclimation period, each fish species (*S. carolitertii* and *S. torgalensis*) was exposed (30 days) to four different experimental conditions, under a 2 × 2 factorial design: i) control (19 and 23 °C, respectively, pH 6.9 and 7.3 for both species); ii) warming (22 and 26 °C, respectively, pH 6.9 and 7.3 for both species); iii) acidification (19 and 23 °C, respectively, pH 6.5 and 6.9 for both species); iv) combined warming and acidification scenario (22 and 26 °C, respectively, pH 6.5 and 6.9 for both species). Warming and acidification conditions were accomplished in order to experimentally assess the responses of each fish species to the tested climate change scenarios (temperature increase = + 3 °C; ΔpH = − 0.4), based on the IPCC's RCP 6.0 scenario (Field et al., 2014). During laboratory acclimation and experimental exposure, a mixture of bloodworms/white mosquito larvae (TMC Iberia, Portugal) and *Spirulina* spp. (flake food, Ocean nutrition, Belgium) was provided ad libitum to fish, on a daily basis. Light regime was set to 12 h:12 h (light/dark cycle), in accordance to prevailing natural light conditions. Monitoring of nitrate, nitrite and ammonia levels was performed daily, using colorimetric tests (Profi Test, Salifert, Holland), with abiotic parameters being kept below detectable levels, during the entire experimental procedure. Monitoring of dissolved oxygen and pH was performed through an automatic control device (Profilux 3.1N, GHL, Germany), with set point values being adjusted and monitored automatically. Individual oxygen (PL-0368, GHL, Germany) and pH (PL-0071, GHL, Germany) sensors were used. Conductivity levels were continuously (Profilux 3.1N, GHL, Germany) and individually (PL-0055, GHL, Germany) monitored, while being kept at 400 to 500 $\mu S{\cdot}cm^{-1}$. Additional daily conductivity checks were performed, using handheld monitoring equipment (CO30, VWR, Portugal). Programmable dosing systems (Easy Dose 3, TMC Iberia, Portugal) connected to indoors-freshwater tanks (300 or 600 $\mu S{\cdot}cm^{-1}$), allowed inflow of freshwater to experimental tanks, in order to maintain conductivity levels within desired range (400–500 $\mu S{\cdot}cm^{-1}$). Maintenance of dissolved oxygen/pH values was accomplished, as follows: injection of certified $N_2/CO_2$ (Air Liquide, Portugal) to down regulate values and aeration with atmospheric filtered air (soda lime, Sigma-Aldrich) to up regulate. All water parameters for the different experimental treatments are shown in Table 1.

After experimental exposure, a set number of fish ($n$ = 6), derived

**Table 1**
Results of two-way ANOVA performed in order to assess the effects of temperature (Temp) and pH on the activity of each metabolic enzymes (lactate dehydrogenase (LDH) and citrate synthase (CS)) of *Squalius carolitertii* and *S. torgalensis*, following an exposure of 30 days to conditions simulating present day and future climate change scenarios. Significant values ($p < 0.013$) are highlighted in bold.

| *S. carolitertii* | | |
|---|---|---|
| | F | *p*-Value |
| LDH | | |
| Temp | 15.764 | **0.001** |
| pH | 12.289 | **0.004** |
| Temp ∗ pH | 20.245 | < **0.001** |
| CS | | |
| Temp | 0.524 | 0.478 |
| pH | 33.461 | < **0.001** |
| Temp ∗ pH | 0.947 | 0.343 |

| *S. torgalensis* | | |
|---|---|---|
| | F | *p*-Value |
| LDH | | |
| Temp | 0.503 | 0.487 |
| pH | 15.832 | **0.001** |
| Temp ∗ pH | 48.279 | < **0.001** |
| CS | | |
| Temp | 0.176 | 0.680 |
| pH | 3.060 | 0.096 |
| Temp ∗ pH | 4.103 | 0.057 |

from each treatment and species, was euthanized and the collected samples were immediately frozen in liquid nitrogen and stored at − 80 °C for biochemical analyses. All experimental procedures were performed under EU compulsory requirements/guidelines (Directive 2010/63/EU, 22nd September 2010) for animal's protection for scientific purposes (ORBEA – Animal Welfare Body of FCUL Statement 5/2016).

### 2.3. Metabolic enzyme activity

Maximum activity levels of citrate synthase (CS) and lactate dehydrogenase (LDH) were estimated in muscle of both species ($n = 6$ specimens per treatment). CS was used as a marker of the aerobic metabolism (acid citric cycle), while LDH was used as a marker of the anaerobic metabolism (fermentation). CS and LDH determinations were performed based on an adaptations of Driedzic and Almeida-Val (1996) and Rosa et al. (2009). Samples were first homogenized (154 ± 25.8 mg of muscle tissue) in a buffer containing 150 mM imidazole, 1 mM EDTA at pH 7.4 in a glass/PTFE potter–Elvehjem tissue grinder (Kartell, Italy) kept on ice. Homogenates were then centrifuged at 10,000*g* for 10 min at 4 °C. LDH activity was assayed using 1 mM pyruvate as substrate in a buffer containing 0.15 mM NADH, 50 mM imidazole and 1 mM EDTA at pH 7.4. CS activity was assayed in a buffer containing 0.25 mM DTNB, 75 mM Trisbase, and 0.4 mM acetyl CoA at pH 8.0, and the reactions were initiated by adding 0.5 mM oxaloacetate. LDH activity was measured following the oxidation of NADH (extinction coefficient of 6220 $M^{-1}$ $cm^{-1}$) at 340 nm while CS activity was determined based on the reaction of coenzyme A with DTNB (5,5 V dithio-bis (2-nitrobenzoic acid)) at 412 nm (extinction coefficient of 13,600 $M^{-1}$ $cm^{-1}$). Changes in absorbance were measured at 20 °C during 1 min, using a Shimadzu UV-1800 spectrophotometer (Shimadzu Scientific Instruments, Japan). For both enzymes, each sample was run in triplicate (technical replicates). The enzyme results were normalized by measuring the total protein content of the samples according to the Bradford method (Bradford, 1976).

### 2.4. Heat shock response, antioxidant enzymes activities and peroxidative damage

#### 2.4.1. Preparation of tissue extracts

Muscle samples ($n = 6$ per treatment; 154 ± 25.8 mg of muscle tissue) were homogenized (Ultra-Turrax, Ika, Staufen, Germany) in accordance to body mass of each sample in homogenization buffer [300 mg tissue per 1 mL phosphate-buffered saline solution (PBS, pH 7.4): 0.14 M NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$, 1.47 mM $KH_2PO_4$]. All homogenates were then centrifuged (20 min at 14,000 × *g* at 4 °C) and the HSR, antioxidant enzyme activities and lipid peroxidation were quantified in the supernatant fraction as described below. All enzyme assays were tested with commercial enzymes obtained from Sigma (Missouri, USA), and each sample was run in triplicate (technical replicates). The enzyme results were normalized by measuring the total protein content of the samples according to the Bradford method (Bradford, 1976).

#### 2.4.2. Heat shock response

HSP content (HSC70/HSP70, two well studied proteins under several stress conditions) was assessed by ELISA (Enzyme-Linked Immunoabsorbent Assay) as previously described by Rosa et al. (2014). Briefly, a total of 10 µL of homogenate supernatant was diluted in 990 µL of PBS, and 50 µL of the diluted sample was added to 96-well microplates MICROLON600 (Greiner Bio-One GmbH, Germany) and incubated overnight at 4 °C. Microplates were washed on the next day in 0.05% PBS-Tween-20. Subsequently, 100 µL of blocking solution (1% Bovine Serum Albumin, BSA) was added to each well. The microplates were then incubated for 2 h at room temperature in darkness. Then, 50 µL of a solution of 5 µg $mL^{-1}$ primary antibody anti-HSP70/HSC70 (that detects both 72 and 73 kDa proteins, which corresponds to the molecular mass of inducible HSP70 and constitutive HSC70, respectively) was added to each well. Plates were then incubated overnight at 4 °C. The non-linked antibodies were removed by repeating the abovementioned washing method, microplates were then incubated for 90 min at 37 °C with 50 µL of the secondary antibody [anti-mouse IgG Fab specific, ALP conjugate (1 µg $mL^{-1}$) from Sigma-Aldrich (Germany)]. After another wash, 100 µL of substrate *p*-nitrophenyl phosphate tablets (Sigma-Aldrich, Germany) were added to each well and the microplates were incubated at room temperature (10 to 30 min). Finally, 50 µL of stop solution (3 M NaOH) was added to each well and the absorbance was read at 405 nm in a 96-well microplate reader (UVM 340, Biochrom, USA). The amount of HSP70/HSC70 in the samples was calculated from a standard curve of absorbance based on serial dilutions (from 0 to 2000 µg $mL^{-1}$) of purified HSP70 active protein (Acris, USA). HSP70/HSC70 concentrations are presented as µg $mg^{-1}$ total protein.

#### 2.4.3. Antioxidant enzymes activities

Glutathione *S*-transferase (GST; an enzyme that convert xenobiotics into other less toxic conjugates) total activity (EC 2.5.1.18) was determined according to Habig et al. (1974) and optimized for 96-well microplate (Sigma Technical Bulletin, CS0410). This procedure measure the conjugation of the thiol group of glutathione to the 1-chloro-2,4-dinitrobenzene (CDNB) substrate. To perform the assay, aliquots (20 µL) from the supernatant fraction of each sample and 180 µL of substrate solution (Dulbecco's Phosphate Buffered Saline with 200 mM L-glutathione reduced and 100 mM 1-chloro-2,4-dinitrobenzene (CDNB) solution, all from Sigma-Aldrich, Germany), were added to 96-well microplates (Nunc-Roskilde, Denmark) and the enzymatic activity determined spectrophotometrically every minute for 6 min at 340 nm, using a microplate reader (UVM 340, Biochrom, USA). Thereby, the increase in absorbance is directly proportional to GST activity. GST activity was calculated using a molar extinction coefficient for CDNB of 5.3 ℰmM (Sigma Technical Bulletin, CS0410). The results are expressed as nmol $min^{-1}$ $mg^{-1}$ total protein.

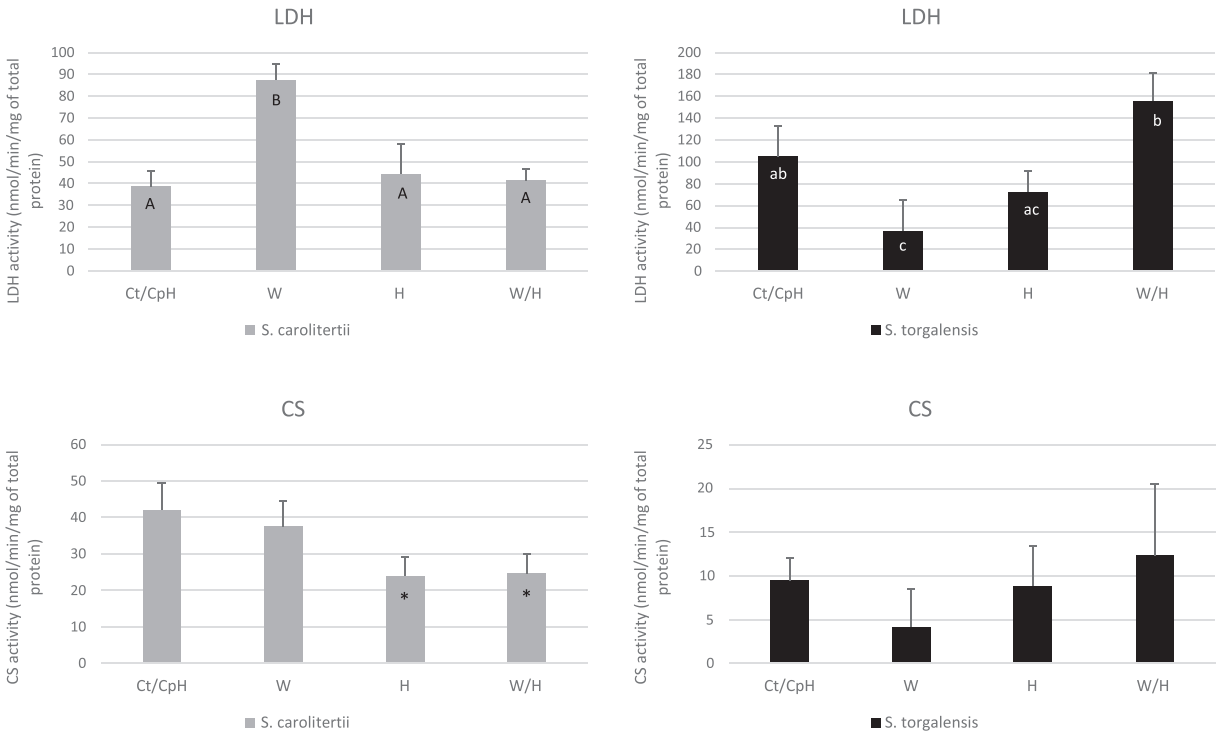


**Fig. 1.** Activity of metabolic enzymes: A) lactate dehydrogenase (LDH, nmol $min^{-1}$ $mg^{-1}$ of total protein), and B) citrate synthase (CS, nmol $min^{-1}$ $mg^{-1}$ of total protein) in the muscle of *Squalius carolitertii* and *S. torgalensis* exposed for 30 days to control temperature (Ct) and pH (CpH), warming (W; + 3 °C) and acidification (H; ΔpH = 0.4). Values represent mean ± SD ($n = 6$). Different letters represent significant differences between treatments ($p < 0.013$). Asterisks represent significant differences between pH within the same temperature ($p < 0.013$).

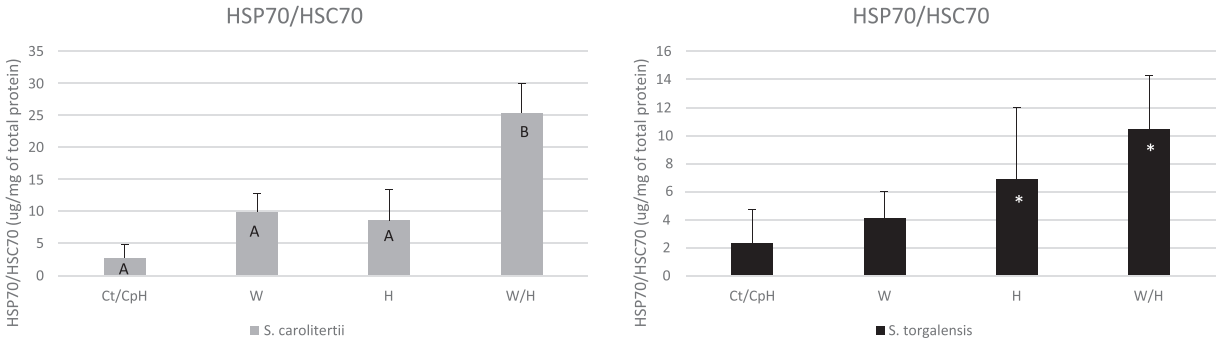


**Fig. 2.** Concentration of heat shock proteins (HSP, μg $mg^{-1}$ of total protein) in the muscle of *Squalius carolitertii* and *S. torgalensis* exposed for 30 days to control temperature (Ct) and pH (CpH), warming (W; + 3 °C) and acidification (H; ΔpH = 0.4). Values represent mean ± SD ($n = 6$). Different letters represent significant differences between treatments ($p < 0.013$). Asterisks represent significant differences between pH within the same temperature ($p < 0.013$).

Catalase (an enzyme that catalyzes the decomposition of hydrogen peroxide to water and oxygen) activity was assessed through an adaptation to the method described by Johansson and Borg (1988). In this assay, 20 μL of each sample, 100 μL of 100 mM Potassium phosphate and 30 μL of methanol were added to a 96-well microplate, which was promptly shaken and incubated for 20 min. Afterwards, 30 μL of potassium hydroxide (10 M KOH) and 30 μL of purpald (34.2 mM in 0.5 M HCl) were added to each well, and the plate shaken and incubated for another 10 min. Subsequently, 10 μL of potassium periodate (65.2 mM in 0.5 M KOH) was added to each well and a final incubation was performed, for 5 min. Using a microplate reader (UVM 340, Biochrom, USA), enzymatic activity was determined spectrophotometrically at 540 nm. Formaldehyde concentration of the samples was calculated based on a calibration curve (from 0 to 75 μM formaldehyde), followed by the calculation of the CAT activity of each sample, were one unit of catalase is defined as the amount that causes the formation of 1.0 nmol of formaldehyde per minute at 25 °C. The results are expressed in relation to total protein content (nmol $min^{-1}$ $mg^{-1}$ protein).

The Superoxide dismutase (SOD; an enzyme that catalyzes the dismutation of the superoxide radical into oxygen or hydrogen peroxide) assay follows the nitrobluetetrazolium (NBT) method adapted from (Sun et al., 1988). In this assay, 10 μL of SOD standard or sample were added to a 96-well microplate (Nunc Roskilde, Denmark), followed by the addition of 200 μL of 50 mM phosphate buffer (pH 8.0) (Sigma-Aldrich, Germany), 10 μL of 3 mM EDTA (Riedel-de Haën, Germany), 10 μL of 3 mM xanthine (Sigma-Aldrich, Germany) and10 μL of 0.75 mM NBT (Sigma-Aldrich, Germany) to each well. The reaction was started with the addition of 100 mU XOD (Sigma-Aldrich, Germany), and the absorbance recorded every 5 min for 25 min, at 550 nm, using a microplate reader (UVM340, Biochrom, USA). Negative control included all components except SOD or sample and produced a maximal increase in absorbance at 560 nm. This allowed determining the inhibition percentage per minute. SOD from bovine erythrocytes (Sigma-Aldrich, Germany) was used as standard and positive control. The total SOD activity was expressed in % of inhibition $mg^{-1}$ total protein.

**Table 2**
Results of two-way ANOVA performed in order to assess the effects of temperature (Temp) and pH on the activity of heat shock proteins (HSP), each antioxidant enzymes (glutathione *S*-transferase (GST), superoxide dismutase activity (SOD) and catalase (CAT)) and malondialdehyde (MDA) of *Squalius carolitertii* and *S. torgalensis* following an exposure of 30 days to conditions simulating present day and future climate change scenarios. Significant values ($p < 0.013$) are highlighted in bold.

| *S. carolitertii* | | |
|---|---|---|
| | F | *p*-Value |
| HSP | | |
| Temp | 50.705 | < **0.001** |
| pH | 40.531 | < **0.001** |
| Temp ∗ pH | 8.339 | **0.011** |
| GST | | |
| Temp | 1.428 | 0.248 |
| pH | 0.185 | 0.672 |
| Temp ∗ pH | 0.105 | 0.750 |
| CAT | | |
| Temp | 0.061 | 0.808 |
| pH | 0.284 | 0.601 |
| Temp ∗ pH | 0.139 | 0.714 |
| SOD | | |
| Temp | 4.333 | 0.054 |
| pH | 2.717 | 0.119 |
| Temp ∗ pH | 3.956 | 0.064 |
| MDA | | |
| Temp | 0.019 | 0.891 |
| pH | 2.835 | 0.109 |
| Temp ∗ pH | 1.620 | 0.219 |

| *S. torgalensis* | | |
|---|---|---|
| | F | *p*-Value |
| HSP | | |
| Temp | 3.839 | 0.063 |
| pH | 15.554 | **0.001** |
| Temp ∗ pH | 0.436 | 0.516 |
| GST | | |
| Temp | 0.224 | 0.641 |
| pH | 1.490 | 0.235 |
| Temp ∗ pH | 0.683 | 0.417 |
| CAT | | |
| Temp | 0.406 | 0.531 |
| pH | 0.241 | 0.629 |
| Temp ∗ pH | 2.054 | 0.167 |
| SOD | | |
| Temp | 0.039 | 0.846 |
| pH | 18.804 | < **0.001** |
| Temp ∗ pH | 7.258 | 0.014 |
| MDA | | |
| Temp | 1.375 | 0.254 |
| pH | 2.703 | 0.114 |
| Temp ∗ pH | 4.714 | 0.041 |

#### 2.4.4. Lipid peroxide assay (malondialdehyde concentration)

Lipid peroxide assay was determined according to the thiobarbituric acid reactive substances (TBARS) protocol, adapted from Uchiyama and Mihara (1978), though the quantification of a specific end-product of the oxidative degradation process of lipids, malondialdehyde (MDA). Aliquots (5 μL) of each sample were added to 45 μL of 50 mM monobasic sodium phosphate buffer in a microtube. Following this, 12.5 μL of sodium dodecyl sulfate (8.1%), 93.5 μL of trichloroacetic acid (20%, pH 3.5) and 93.5 μL of thiobarbituric acid (1%) were added to each microtube. Afterwards, 50.5 μL of ultrapure water was added to this mixture and vortexed for 30 s and the microtube lids punctured just before the incubation in boiling water, for 10 min, after which they were allowed to cool on ice. Subsequently, 62.5 μL of ultrapure water and 312.5 μL of *n*-butanol pyridine (15:1, v/v) (Sigma-Aldrich, Germany) were added and microtubes were centrifuged (5000 × *g*; 5 min.). Duplicates of 150 μL of the supernatant of each reaction were put into a 96-well microplate (Nunc Roskilde, Denmark) and absorbance was measured at 532 nm. To quantify the lipid peroxides, an eight-point calibration curve (0–0.3 μM TBARS) was calculated using malondialdehyde (dimethylacetal; MDA; Merck, Switzerland) standards. MDA values are expressed as nmol $mg^{-1}$ total protein.

### 2.5. Statistical analyses

In order to infer the statistical significance of warming and acidification in underlying metabolic (i.e. CS and LDH) and antioxidant stress responses (i.e. HSP, GST, SOD, CAT and MDA), a two-way MANOVA was performed for each group. As MANOVA revealed significant differences, two-way ANOVA followed by Tukey *post hoc* tests were performed whenever the interaction between temperature and pH was significant, to understand the effect of explaining variables on each enzyme. In these analyses, the Dunn-Sidak correction was applied in order to adjust associated significance level of the family-wise type-I error (Quinn and Keough, 2002). A total of 4 comparisons were applied (2 temperature levels combined with 2 pH values), resulting in a significance level of 0.013. Prior to all performed analyses, data was checked for normality and homocedascity using Shapiro-Wilk's and Levene's tests, respectively. Unless stated otherwise, a significant level of 0.05 was used. All statistical analyses were performed, using Statistica 7.0 software (StatSoft Inc., USA).

## 3. Results

### 3.1. Metabolic enzymes

Acidification caused significant changes in the metabolic enzyme activities of *S. carolitertii* and *S. torgalensis*, with a significant interaction with temperature (two-way MANOVA: $p < 0.05$; Table S1). Regarding the former species, LDH activity was significantly affected by both variables, together with a significant interaction between temperature and pH (two-way ANOVA: $p < 0.013$; Table 1). In fact, increasing temperature prompted an increase in the activity of LDH but only under normocapnia (Tukey *post hoc* test: $p < 0.013$; Fig. 1A). On the other hand, CS activity was only significantly affected by pH with a significant decrease of its activity in organisms exposed to water acidification (two-way ANOVA: $p < 0.013$; Fig. 1B; Table 1). Regarding *S. torgalensis*, LDH activity was significantly affected by pH and by its interaction with temperature (two-way ANOVA: $p < 0.013$; Table 1). In more detail, individuals exposed to the combined warming and acidification showed significantly higher LDH activity (Tukey *post hoc* test: $p < 0.013$; Fig. 1A). Moreover, under normocapnia, LDH activity was significantly decreased by increasing temperature (Tukey *post hoc* test: $p < 0.013$; Fig. 1A). As for CS activity, neither temperature nor pH exerted a significant effect (two-way ANOVA: $p > 0.013$; Fig. 1B; Table 1).

### 3.2. Heat shock response, antioxidant enzymes activities and peroxidative damage

The heat shock and antioxidant (i.e. GST, CAT and SOD) responses, as well as cellular damage assessed in *S. carolitertii* were significantly influenced by both warming and acidification together with a significant interaction between the explaining variables (two-way MANOVA: $p < 0.05$; Table S1). However, only HSP content showed significant differences between the experimental conditions (Fig. 2 and Table 2). In more detail, individuals exposed to conditions simulating a future climate change scenario (i.e. warming and acidification tested together) showed a significant increase in HSP content when compared with the ones exposed to the other conditions (Tukey *post hoc* test:

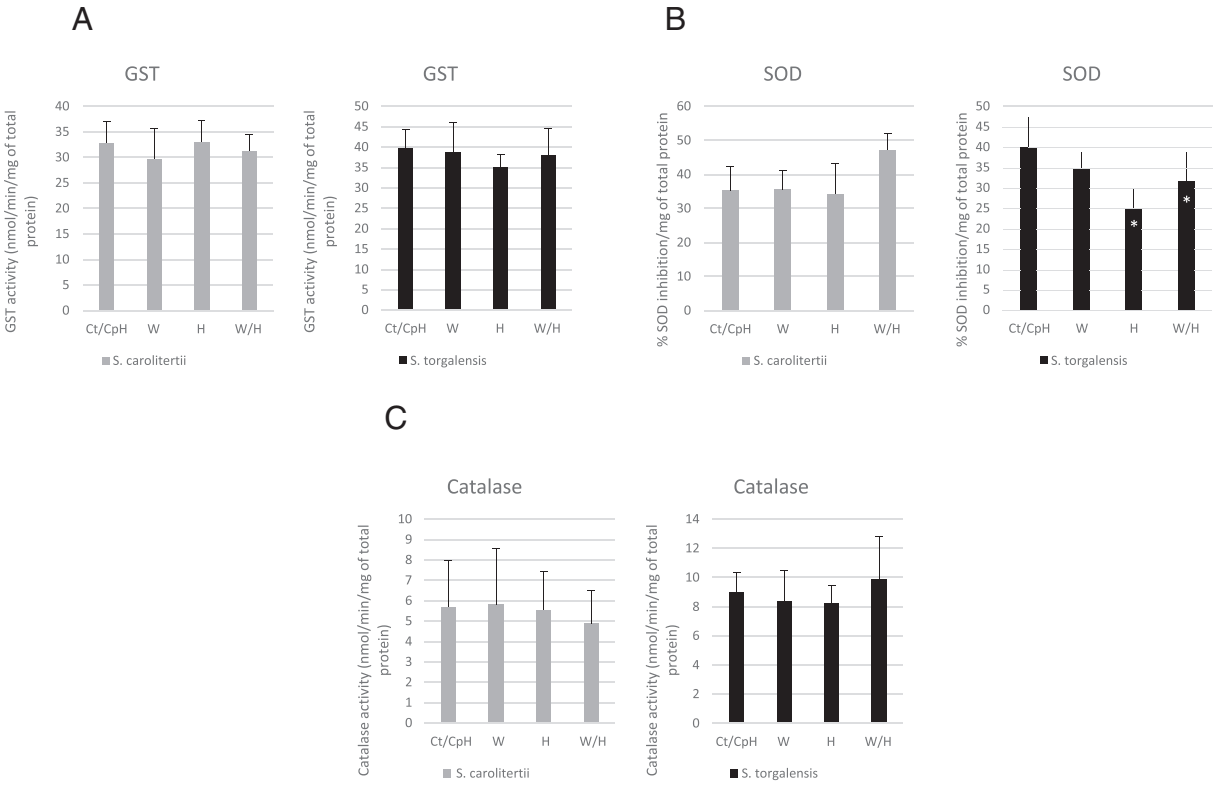


**Fig. 3.** Activity of antioxidant enzymes: A) Glutathione *S*-transferase (GST, nmol $min^{-1}$ $mg^{-1}$ of total protein), B) percentage inhibition of superoxide dismutase (SOD, % inhibition $mg^{-1}$ of total protein) and C) catalase (CAT, nmol $min^{-1}$ $mg^{-1}$ of total protein) in the muscle of *Squalius carolitertii* and *S. torgalensis* exposed for 30 days to control temperature (Ct) and pH (CpH), warming (W; + 3 °C) and acidification (H; ΔpH = 0.4). Values represent mean ± SD ($n$ = 6). Asterisks represent significant differences between pH within the same temperature ($p$ < 0.013).

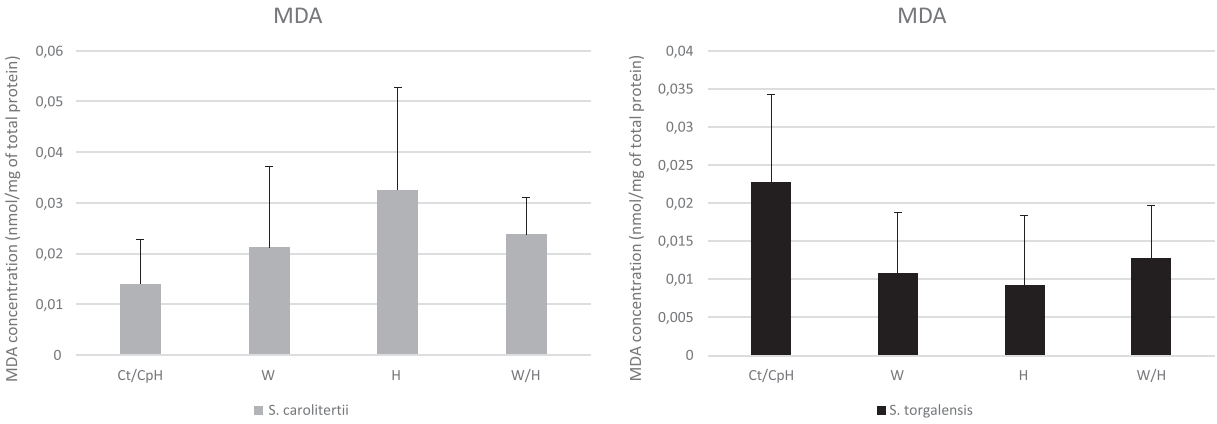


**Fig. 4.** Concentration of malondialdehyde (MDA, nmol $mg^{-1}$ of total protein) in the muscle of *Squalius carolitertii* and *S. torgalensis* exposed for 30 days to control temperature (Ct) and pH (CpH), warming (W; + 3 °C) and acidification (H; ΔpH = 0.4). Values represent mean ± SD ($n$ = 6).

$p$ < 0.013; Fig. 2). Regarding *S. torgalensis*, pH and the interaction of this factor with temperature significantly affected heat shock, antioxidant, and peroxidative damage responses (two-way MANOVA: $p$ < 0.05; Table S1), with no significant effect of temperature (two-way MANOVA: $p$ > 0.05; Table S1). Looking into more detail to each response, HSP concentration significantly increased under the acidification condition (two-way ANOVA: $p$ < 0.013; Table 2) whereas SOD inhibition decreased significantly in the acidification condition (two-way ANOVA: $p$ < 0.013; Table 2). As for the other endpoints (i.e. GST, CAT and MDA), neither warming nor acidification significantly affected their activity/concentration (two-way ANOVA: $p$ > 0.013; Figs. 3 and 4; Table 2).

## 4. Discussion

Even though freshwater ecosystems are considered to be extremely vulnerable to environmental changes (Field et al., 2014), the physiology of freshwater fishes under the context of climate change is poorly known (Mccairns et al., 2016; Ou et al., 2015b). This study reports the first results on the impact of climate change related variables in the metabolism and oxidative stress enzymatic machinery of two species of the genus *Squalius* inhabiting different climatic regions.

Our results showed that conditions simulating a climate change scenario affected the enzymatic activity of citrate synthase (CS) and lactate dehydrogenase (LDH) on *S. carolitertii* and *S. torgalensis*. For both species, increasing temperature under normocapnia, elicited changes in LDH activity, causing an increase in its activity in *S. carolitertii* with an

opposite trend in *S. torgalensis*. Although not significantly different from the control condition, under the combined effects of warming and acidification, *S. torgalensis* showed a trend in increased LDH activity that was not seen in *S. carolitertii*, suggesting the existence of a synergistic effect between these two climate change related variables in this population. On the other hand, acidification caused a significant decrease in CS activity of *S. carolitertii*, contrary to *S. torgalensis* which was not affected by conditions simulating climate change.

Typically, LDH activity increases with stressful conditions (e.g. high temperatures, scarcity of oxygen) and CS activity diminishes when oxygen is scarce (Almeida-Val et al., 2000; Campos et al., 2017; Rosa et al., 2016; Storey and Storey, 2005). Hence, these results suggest that LDH activity of *S. carolitertii* was increased to compensate for the increase in the metabolic rate due to the higher temperature. On the other hand, the CS activity in *S. carolitertii* decreased as the result of pH increase. The increase in LDH activity (marker for anaerobic metabolism) and decrease in CS activity (marker for aerobic metabolism), albeit in different conditions, may not be a viable long-term response, given that the anaerobic metabolism requires finite fermentable subtracts and leads to cytotoxicity (Rosa et al., 2016; Rosa and Seibel, 2008). On the other hand, *S. torgalensis* did not increase LDH or decrease CS levels under higher temperatures, suggesting that there are no increases in both aerobic and anaerobic metabolism. Thus, it seems that *S. torgalensis* can maintain its metabolic homeostasis, being able to cope with effects of climate changes. Moreover, when exposed to increased temperature (under control pH), the anaerobic potential is reduced (rather than increasing as it would be expected in a more stressful condition). This might be a result of the adaptation of this species to higher temperatures, since it is usually exposed to high temperatures (e.g. 38 °C) during the summer season, where individuals of this species may be confined to small pools (Jesus et al., 2013). These results are in agreement with previous transcriptomic studies on both species, which suggest that *S. torgalensis* may be better suited to deal with adverse environmental conditions (Jesus et al., 2016, 2017). In these studies, a similar trend was found, with increased *cs* gene expression being favored in relation to increases in *ldha* gene expression by *S. torgalensis* (Jesus et al., 2017, 2016). The performance of this species under stressful conditions may be the result of its adaptation to harsher environmental conditions giving it needs to cope with a wider range of environmental conditions.

In general, while the oxidative stress of both *S. carolitertii* and *S. torgalensis* were not extremely affected by the temperature and pH, the HSR was significantly impacted. Regarding the HSR, conditions simulating a future scenario of climate change significantly affected both species, although in different ways. While HSP concentration of *S. carolitertii* significantly increased under the combined warming and acidification condition, suggesting a synergistic effect of temperature and pH, HSP concentration of *S. torgalensis* was only affected by pH, regardless of the temperature to which fish were exposed. These results raise concerns regarding the persistence of both species, with particular emphasis for *S. carolitertii*, which showed the greatest increase in HSP concentration under the synergistic scenario. In fact, maintaining elevated levels of HSP for extended periods of time may be disadvantageous since the resources may be relocated from other key biological processes (e.g. growth, energy production) to the refolding of denatured proteins (Sorensen et al., 2003; Veilleux et al., 2015). Once again, our results are in line with previous gene expression studies on these species, which demonstrated that *S. torgalensis* presents a better fine-tuned HSR than *S. carolitertii*, both during short (Jesus et al., 2016, 2013) and longer exposure periods (Jesus et al., 2017).

Interestingly, none of the antioxidant enzymes was significantly affected by any tested condition, except for SOD activity of *S. torgalensis* that significantly decreased with acidification regardless of the temperature to which fish were exposed. These results, along with the absence of changes in lipid peroxidation, indicate that both species were not under oxidative stress in the projected climate change conditions. These results are in contrast with what was found for other species, subject to similar conditions, where significant changes in antioxidant enzymes and lipid peroxidation was found [e.g. (Pimentel et al., 2015; Rosa et al., 2016, 2014)]. However, freshwater fish are usually exposed to higher temperatures and pH variations (daily and throughout the year) than seawater animals and thus the increase of 3 °C and the decrease of 0.4 units on pH was not enough to induce an oxidative stress response.

Overall, our results, suggest that *S. carolitertii* may struggle to cope with future climate change, particularly due to the effects of warming and acidification in metabolic activity and heat shock response. On the other hand, *S. torgalensis* seem to be better suited to these changes. Nevertheless, this species may still be at risk mainly due to the inability to maintain the trade-off between the upregulation of HSP and its costs. This study is of utmost importance to better comprehend how freshwater fishes will cope with future climate change and for the adoption of proper conservation strategies, which is particularly relevant for species such as *S. torgalensis*, considered a critically endangered species. Although our results do not raise a serious concern on the future of these species, further studies should focus on the combined effects of warming and acidification (Pimentel et al., 2015; Rosa et al., 2014, 2016; Jesus et al., 2017, under review) together with other climate change related variables (Crozier et al., 2008; Prado-Lima and Val, 2016).

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## References

Almeida-Val, V.M.F., Val, A.L., Duncan, W.P., Souza, F.C.A., Paula-Silva, M.N., Land, S., 2000. Scaling effects on hypoxia tolerance in the Amazon fish *Astronotus ocellatus* (Perciformes: Cichlidae): contribution of tissue enzyme levels. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 125, 219–226. http://dx.doi.org/10.1016/S0305-0491(99)00172-8.

Angilletta, M.J., 2002. The evolution of thermal physiology in endotherms. J. Therm. Biol. 27, 249–268.

Aurélio, M., Faleiro, F., Lopes, V.M., Pires, V., Lopes, A.R., Pimentel, M.S., Repolho, T., Baptista, M., Narciso, L., Rosa, R., 2013. Physiological and behavioral responses of temperate seahorses (*Hippocampus guttulatus*) to environmental warming. Mar. Biol. 160, 2663–2670. http://dx.doi.org/10.1007/s00227-013-2259-8.

Berg, M.P., Toby Kiers, E., Driessen, G., van der Heijden, M., Kooi, B.W., Kuenen, F., Liefting, M., Verhoef, H.A., Ellers, J., 2010. Adapt or disperse: understanding species persistence in a changing world. Glob. Chang. Biol. 16, 587–598. http://dx.doi.org/10.1111/j.1365-2486.2009.02014.x.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein dye binding. Anal. Biochem. 72, 248–254. http://dx.doi.org/10.1016/0003-2697(76)90527-3.

Campos, D.F., Jesus, T.F., Kochhann, D., Heinrichs-Caldas, W., Coelho, M.M., Almeida-Val, V.M.F., 2017. Metabolic rate and thermal tolerance in two congeneric Amazon fishes: *Paracheirodon axelrodi* Schultz, 1956 and *Paracheirodon simulans* Géry, 1963 (Characidae). Hydrobiologia 789, 133–142. http://dx.doi.org/10.1007/s10750-016-2649-2.

Carvalho, S.B., Brito, J.C., Crespo, E.J., Possingham, H.P., 2010. From climate change predictions to actions - conserving vulnerable animal groups in hotspots at a regional scale. Glob. Chang. Biol. 16, 3257–3270. http://dx.doi.org/10.1111/j.1365-2486.2010.02212.x.

Coelho, M.M., Bogutskaya, N.G., Rodrigues, J.A., Collares-Pereira, M.J., 1998. *Leuciscus torgalensis* and *L. aradensis*, two new cyprinids for Portuguese fresh waters. J. Fish Biol. 52, 937–950.

Collins, M., Knutti, R., Arblaster, J., Dufresne, J.-L., Fichefet, T., Friedlingstein, P., Gao, X., Gutowski, W.J., Johns, T., Krinner, G., Shongwe, M., Tebaldi, C., Weaver, A.J., Wehner, M., 2013. Long-term climate change: projections, commitments and irreversibility. In: Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change, pp. 1029–1136. http://dx.doi.org/10.1017/CBO9781107415324.024.

Crozier, L.G., Hendry, A.P., Lawson, P.W., Quinn, T.P., Mantua, N.J., Battin, J., Shaw, R.G., Huey, R.B., 2008. PERSPECTIVE: potential responses to climate change in

organisms with complex life histories: evolution and plasticity in Pacific salmon. Evol. Appl. 1, 252–270. http://dx.doi.org/10.1111/j.1752-4571.2008.00033.x.
Doadrio, I., 1988. *Leuciscus carolitertii* n.sp. from the Iberian Peninsula (Pisces: Cyprinidae). Senckenb. Biol. 68, 301–309.
Dong, Y., Miller, L.P., Sanders, J.G., Somero, G.N., 2008. Heat-shock protein 70 (Hsp70) expression in four limpets of the genus *Lottia*: interspecific variation in constitutive and inducible synthesis correlates with in situ exposure to heat stress. Biol. Bull. 215, 173–181 (doi:215/2/173 [pii]).
Driedzic, W.R., Almeida-Val, V.M., 1996. Enzymes of cardiac energy metabolism in Amazonian teleosts and the fresh-water stingray (*Potamotrygon hystrix*). J. Exp. Zool. 274, 327–333. http://dx.doi.org/10.1002/(SICI)1097-010X(19960415)274:6<327::AID-JEZ1>3.0.CO;2-Q.
Fangue, N.A., Hofmeister, M., Schulte, P.M., 2006. Intraspecific variation in thermal tolerance and heat shock protein gene expression in common killifish, Fundulus Heteroclitus. J. Exp. Biol. 209, 2859–2872. http://dx.doi.org/10.1242/jeb.02260.
Field, C.B., Barros, V.R., Dokken, D.J., Mach, K.J., Mastrandrea, M.D., Bilir, T.E., Chatterjee, M., Ebi, K.L., Estrada, Y.O., Genova, R.C., Girma, B., Kissel, E.S., Levy, A.N., MacCracken, S., Mastrandrea, P.R., White, L.L. (Eds.), 2014. Climate Change 2014: Impacts, Adaptation, and Vulnerability. Part A: Global and Sectoral Aspects. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change, in: Intergovermental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, pp. 1132.
Füssel, H., Jol, A., Kurnik, B., Hemming, D., 2012. Climate change, impacts and vulnerability in Europe 2012: an indicator-based report. EEA Report. http://dx.doi.org/10.2800/66071.
Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139. http://dx.doi.org/10.1017/CBO9781107415324.004.
Hartmann, D.L., Tank, A.M.G.K., Rusticucci, M., 2013. Working Group I Contribution to the IPCC Fifth Assessment Report, Climatie Change 2013: The Physical Science Basis, Intergovermental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.
Hoffmann, A.A., Sgrò, C.M., 2011. Climate change and evolutionary adaptation. Nature 470, 479–485.
Jesus, T.F., Inácio, A., Coelho, M.M., 2013. Different levels of hsp70 and hsc70 mRNA expression in Iberian fish exposed to distinct river conditions. Genet. Mol. Biol. 36, 61–69.
Jesus, T.F., Grosso, A.R., Almeida-Val, V.M.F., Coelho, M.M., 2016. Transcriptome profiling of two Iberian freshwater fish exposed to thermal stress. J. Therm. Biol. 55, 54–61. http://dx.doi.org/10.1016/j.jtherbio.2015.11.009.
Jesus, T.F., Moreno, J.M., Repolho, T., Athanasiadis, A., Rosa, R., Almeida-Val, V.M.F., Coelho, M.M., 2017. Protein analysis and gene expression indicate differential vulnerability of Iberian fish species under a climate change scenario (under review). PLoS One 1–18.
Johansson, L.H., Borg, L.A., 1988. A spectrophotometric method for determination of catalase activity in small tissue samples. Anal. Biochem. 174, 331–336. http://dx.doi.org/10.1016/0003-2697(88)90554-4.
Leduc, A.O.H.C., Munday, P.L., Brown, G.E., Ferrari, M.C.O., 2013. Effects of acidification on olfactory-mediated behaviour in freshwater and marine ecosystems: a synthesis. Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 368, 20120447.
Magalhães, M.F., Schlosser, I.J., Collares-Pereira, M.J., 2003. The role of life history in the relationship between population dynamics and environmental variability in two Mediterranean stream fishes. J. Fish Biol. 63, 300–317.
Mccairns, R.J.S., Smith, S., Sasaki, M., Bernatchez, L., Beheregaray, L.B., 2016. The adaptive potential of subtropical rainbowfish in the face of climate change: heritability and heritable plasticity for the expression of candidate genes. Evol. Appl. 9, 531–545. http://dx.doi.org/10.1111/eva.12363.
McClelland, G.B., Craig, P.M., Dhekney, K., Dipardo, S., 2006. Temperature- and exercise-induced gene expression and metabolic enzyme changes in skeletal muscle of adult zebrafish (*Danio rerio*). J. Physiol. 577, 739–751. http://dx.doi.org/10.1113/jphysiol.2006.119032.
Morris, J.P., Thatje, S., Hauton, C., 2013. The use of stress-70 proteins in physiology: a reappraisal. Mol. Ecol. 44, 1494–1502. http://dx.doi.org/10.1111/mec.12216.
Munday, P.L., Dixson, D.L., Donelson, J.M., Jones, G.P., Pratchett, M.S., Devitsina, G.V., Døving, K.B., 2009. Ocean acidification impairs olfactory discrimination and homing ability of a marine fish. Proc. Natl. Acad. Sci. U. S. A. 106, 1848–1852. http://dx.doi.org/10.1073/pnas.0809996106.
Mwangangi, D.M., Mutungi, G., 1994. The effects of temperature acclimation on the oxygen consumption and enzyme activity of red and white muscle fibres isolated from the tropical freshwater fish *Oreochromis niloticus*. J. Fish Biol. 44 (1033–1), 1033–1043.
de Oliveira, A.M., Val, A.L., 2016. Effects of climate scenarios on the growth and physiology of the Amazonian fish tambaqui (*Colossoma macropomum*) (Characiformes: Serrasalmidae). Hydrobiologia 1–12. http://dx.doi.org/10.1007/s10750-016-2926-0.
Ou, M., Hamilton, T.J., Eom, J., Lyall, E.M., Gallup, J., Jiang, A., Lee, J., Close, D.a., Yun, S.-S., Brauner, C.J., 2015a. Responses of pink salmon to $CO_2$-induced aquatic acidification. Nat. Clim. Chang. http://dx.doi.org/10.1038/nclimate2694.
Ou, M., Hamilton, T.J., Eom, J., Lyall, E.M., Gallup, J., Jiang, A., Lee, J., Close, D.a., Yun, S.-S., Brauner, C.J., 2015b. Responses of pink salmon to $CO_2$-induced aquatic acidification. Nat. Clim. Chang. 5, 950–955. http://dx.doi.org/10.1038/nclimate2694.
Pimentel, M.S., Faleiro, F., Diniz, M., Machado, J., Pousão-Ferreira, P., Peck, M.A., Pörtner, H.O., Rosa, R., 2015. Oxidative stress and digestive enzyme activity of flatfish larvae in a changing ocean. PLoS One 10, 1–18. http://dx.doi.org/10.1371/journal.pone.0134082.
Pimentel, M.S., Faleiro, F., Marques, T., Bispo, R., Dionísio, G., Faria, A.M., Machado, J., Peck, M.A., Pörtner, H., Pousão-Ferreira, P., Gonçalves, E.J., Rosa, R., 2016. Foraging behaviour, swimming performance and malformations of early stages of commercially important fishes under ocean acidification and warming. Clim. Chang. 137, 495–509. http://dx.doi.org/10.1007/s10584-016-1682-5.
Podrabsky, J.E., Somero, G.N., 2004. Changes in gene expression associated with acclimation to constant temperatures and fluctuating daily temperatures in an annual killifish *Austrofundulus limnaeus*. J. Exp. Biol. 207, 2237–2254. http://dx.doi.org/10.1242/jeb.01016.
Pörtner, H.-O., Karl, D.M., Boyd, P.W., Cheung, W.W., Lluch-Cota, S.E., Nojiri, Y., Schmidt, D.N., Zavialov, P.O., 2014. Ocean systems. In: Climate Change 2014: Impacts, Adaptation and Vulnerability. Part A: Global and Sectoral Aspects. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change, pp. 411–484. http://dx.doi.org/10.2134/jeq2008.0015br.
Prado-Lima, M., Val, A.L., 2016. Transcriptomic characterization of tambaqui (*Colossoma macropomum*, Cuvier, 1818) exposed to three climate change scenarios. PLoS One 11, e0152366. http://dx.doi.org/10.1371/journal.pone.0152366.
Quinn, G.P., Keough, M.J., 2002. Experimental Design and Data Analysis for Biologists. Cambridge University Press, UK.
Reusch, T.B.H., Wood, T.E., 2007. Molecular ecology of global change. Mol. Ecol. 16, 3973–3992. http://dx.doi.org/10.1111/j.1365-294X.2007.03454.x.
Rosa, R., Seibel, B.A., 2008. Synergistic Effects of Climate-Related Variables Suggest Future Physiological Impairment in a Top Oceanic Predator.
Rosa, R., Trueblood, L., Seibel, B.A., 2009. Ecophysiological influence on scaling of aerobic and anaerobic metabolism of pelagic gonatid squids. Physiol. Biochem. Zool. 82, 419–429. http://dx.doi.org/10.1086/591950.
Rosa, R., Trübenbach, K., Pimentel, M.S., Boavida-Portugal, J., Faleiro, F., Baptista, M., Dionísio, G., Calado, R., Pörtner, H.O., Repolho, T., 2014. Differential impacts of ocean acidification and warming on winter and summer progeny of a coastal squid (*Loligo vulgaris*). J. Exp. Biol. 217, 518–525. http://dx.doi.org/10.1242/jeb.096081.
Rosa, R., Ricardo Paula, J., Sampaio, E., Pimentel, M., Lopes, A.R., Baptista, M., Guerreiro, M., Santos, C., Campos, D., Almeida-Val, V.M.F., Calado, R., Diniz, M., Repolho, T., 2016. Neuro-oxidative damage and aerobic potential loss of sharks under elevated $CO_2$ and warming. Mar. Biol. 163, 1–10. http://dx.doi.org/10.1007/s00227-016-2898-7.
Sevcikova, M., Modra, H., Slaninova, A., Svobodova, Z., 2011. Metals as a cause of oxidative stress in fish: a review. Vet. Med. (Praha). 56, 537–546. http://dx.doi.org/10.1016/j.aquatox.2014.06.018.
Somero, G.N., 2010. The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine “winners” and “losers”. J. Exp. Biol. 213, 912–920.
Sorensen, J.G., Kristensen, T.N., Loeschcke, V., 2003. The evolutionary and ecological role of heat shock proteins. Ecol. Lett. 6, 1025–1037. http://dx.doi.org/10.1046/j.1461-0248.2003.00528.x.
Storey, K.B., Storey, J.M., 2005. Oxygen limitation and metabolic rate depression. Funct. Metab. 415–442. http://dx.doi.org/10.1002/047167558X.ch15.
Sun, Y., Oberley, L.W., Li, Y., 1988. A simple method for clinical assay of superoxide dismutase. Clin. Chem. 34, 497–500.
Sun, Y., Yin, G., Zhang, J., Yu, H., Wang, X., 2007. Bioaccumulation and ROS generation in liver of freshwater fish, goldfish *Carassius auratus* under HC Orange No. 1 exposure. Environ. Toxicol. 22, 256–263. http://dx.doi.org/10.1002/tox.20262.
Tomanek, L., 2010. Variation in the heat shock response and its implication for predicting the effect of global climate change on species' biogeographical distribution ranges and metabolic costs. J. Exp. Biol. 213, 971–979. http://dx.doi.org/10.1242/jeb.038034.
Uchiyama, M., Mihara, M., 1978. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal. Biochem. 86, 271–278. http://dx.doi.org/10.1016/0003-2697(78)90342-1.
Van De Waal, D.B., Verschoor, A.M., Verspagen, J.M.H., Van Donk, E., Huisman, J., 2010. Climate-driven changes in the ecological stoichiometry of aquatic ecosystems. Front. Ecol. Environ. http://dx.doi.org/10.1890/080178.
Veilleux, H.D., Ryu, T., Donelson, J.M., van Herwerden, L., Seridi, L., Ghosheh, Y., Berumen, M.L., Leggat, W., Ravasi, T., Munday, P.L., 2015. Molecular processes of transgenerational acclimation to a warming ocean. Nat. Clim. Chang. 5, 1074–1078. http://dx.doi.org/10.1038/nclimate2724.
Vinagre, C., Narciso, L., Pimentel, M., Cabral, H.N., Costa, M.J., Rosa, R., 2013. Contrasting impacts of climate change across seasons: effects on flatfish cohorts. Reg. Environ. Chang. 13, 853–859. http://dx.doi.org/10.1007/s10113-012-0376-4.
Wegele, H., Müller, L., Buchner, J., 2004. Hsp70 and Hsp90—a relay team for protein folding. Rev. Physiol. Biochem. Pharmacol. 151, 1–44. http://dx.doi.org/10.1007/s10254-003-0021-1.
Yamashita, M., Hirayoshi, K., Nagata, K., 2004. Characterization of multiple members of the HSP70 family in platyfish culture cells: molecular evolution of stress protein HSP70 in vertebrates. Gene 336, 207–218. http://dx.doi.org/10.1016/j.gene.2004.04.023.